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ScienceDirect
Veterinary Parasitology 152 (2008) 108–115
veterinary parasitology
www.elsevier.com/locate/vetpar

Patent *Toxocara canis* infections in previously exposed and in helminth-free dogs after infection with low numbers of embryonated eggs

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Received 10 July 2007; received in revised form 20 November 2007; accepted 26 November 2007

Abstract

The outcome of *Toxocara canis* infections in the canine host depends on the migratory pathway of parasite larvae (somatic or tracheal) which is considered to be related to the host's age and its immune status. However, field studies attest high prevalences of patent *T. canis* infections in adult animals. The controlled induction of patent infections with low doses of embryonated eggs was investigated in 18 beagles in a 7-month study until their 16th life month. The animals were assigned to three groups, each consisting of three vertically infected dogs (with a short patent infection as pups before anthelmintic treatment) and three helminth-free dogs. At study days 10 and 40, the animals of groups 1 and 3 were given each 100 embryonated *T. canis* eggs. In each case, group 1 was treated 10 days post-infection with Milbemax®, while dogs of group 3 remained untreated. Control group 2 was not experimentally infected but treated as group 1. Two weeks after first egg administration, a sharp increase of specific antibody reactions in ELISA and increased eosinophilic counts indicated larval invasion in all infected dogs. 42–56 days following first infection, patent infections were detected coproscopically in all animals of group 3, but in none of the uninfected dogs (group 2) or the infected and treated dogs (group 1). Following a 3-month observation period, all animals of the three groups were treated with piperazine citrate to eliminate intestinal infections and all were administered 100 embryonated eggs. Subsequently, patent infections developed in animals of all groups: in one of the infected and treated animals of group 1, in five of the so far not infected control group 2 and in four of the dogs with previous patent infections (group 3). Susceptibility to patent infections was not significantly altered in *T. canis*-free dogs compared to dogs with previous patent infection (vertically acquired or experimentally induced). However, dogs of group 1 treated with Milbemax® after repeated egg administration developed a significantly increased resistance to patent infections as compared to control dogs (group 2). Observed prepatency periods were between 40 and 56 days and did not differ in the three groups. Even in urban areas, facing high infection pressure with *Toxocara* eggs maintained by a high dog and fox population, dogs of all ages are at risk to develop patent *T. canis* infections.

Keywords: *Toxocara canis*; Zoonosis; Resistance; Age influence; Patent infection

1. Introduction

Toxocara canis (Werner, 1782) is one of the most frequently found gastrointestinal helminths of domestic and wild canids (Overgaauw, 1997; Maizels et al., 2006) and the agent of Toxocarosis, a serious

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doi:10.1016/j.vetpar.2007.11.022

zoonotic disease in humans (Despommier, 2003). Infection of the canid arises from vertical larval transmission (intrauterine or lactogenic), from ingestion of embryonated eggs from the environment or ingestion of larvae, e.g., from paratenic hosts like rodent prey animals (Overgaauw, 1997; Lloyd, 1998). Infection source, age of the dog, and number of ingested eggs determine the migratory pathway of the parasite in the dog. Hormonal and immunological status, breed and gender are further factors considered critical for the outcome of larval migration (Lloyd, 1998). The *tracheal* migration pattern leads to the development of adult and patent worms in the intestine. This liver-lung-trachea-oesophagus route is mainly attributed to young animals (Greve, 1971). When adult dogs ingest embryonated eggs, the majority of larvae migrate to *somatic* tissues, leading to their accumulation preferentially in muscles and kidneys, liver and central nervous system (Dubey, 1978; Manhardt and Stoye, 1981). Only in a proportion of adult dogs some larvae undergo tracheal migration resulting in intestinal infection (Lloyd, 1998). Specifications of the dog's age at the transition point from tracheal to somatic pathway differ among authors between about 1–2 months (Sprent, 1958; Overgaauw, 1997) and 6 months (Webster, 1956). However, prevalence studies in dogs worldwide have assessed patent infections in adult dogs (Lloyd, 1993; Barutzki and Schaper, 2003; Sager et al., 2006; Martinez-Moreno et al., 2007). For adult canine hosts it is recognised that patent infections can arise from swallowing intestinal *T. canis* stages expelled by puppies or from larvae in consumed paratenic hosts like small prey animals (Warren, 1969; Saeed et al., 2005). A few experiments with small numbers of adult dogs infected with low numbers of embryonated eggs resulted also in patent infections (Dubey, 1978; Maizels and Meghji, 1984). It has been assumed that this tracheal migration might be related to genetically determined susceptibility or hormonal effects (Lloyd, 1998).

With the aim to elucidate the circumstances under which adult dogs acquire patent infections by ingesting embryonated eggs, we investigated the course of experimental *T. canis* infections in immunologically mature dogs with varied histories of previous exposure to the parasite. In consideration of rising zoonotic awareness and demand for regular, anthelmintic control of patent infections in dogs, we also aimed to investigate the effect of a regular anthelmintic treatment strategy on the development of resistance or susceptibility to patent infections.

2. Materials and methods

2.1. Animals

Eight male and ten female, facility-born crossbred (Beagle × 'Niederlaufhund') dogs originating from three litters, all aged 8–10 months at study day 0, were included in the study. Half of the dogs were vertically infected with *T. canis* and had proven patent infections during puppyhood, as demonstrated by worm expulsion after treatment with piperazine citrate (Stricker) at 5 weeks of age. The third litter consisting of nine dogs was kept strictly *T. canis*-free by separation and application of an intensive anthelmintic treatment scheme: the bitch was administered Milbemax® 20 and 8 days ante partum and 23 days post-partum. Her offspring were treated 2 and 4 weeks post-partum and then monthly with Milbemax® (tablets S, later M according to their weight). From their 4th life week on, these dogs were monitored coproscopically on a monthly basis and had never shown positive results prior to the beginning of the study. All animals were kept in groups of two to four dogs of the same sex, housed in kennels of 6.5 m^2 with 8 m^2 runouts/two dogs, both with concrete floors and wooden stands for rest, cleaned daily with high pressure cleaner at 80 °C (Kärcher). They were fed commercially prepared dried dog food and had access to tap water *ad libitum*. Due to possible contamination with faeces containing worm eggs, the positive control dogs (group 3, described below) were kept isolated from all other animals in a part of the building only accessible for a restricted number of persons after changing clothes and boots. For cages and boots, an ovocide disinfectant solution (Neopredisan 135-1, 2%, Vital Ag, Switzerland) was used in addition to daily high pressure steam cleaning. Once they started shedding eggs, the dogs were washed entirely with a shampoo (Johnson) every second week to minimise reinfection risk through eggs sticking on the hair coat.

Before the experiment started, all dogs received anthelmintic treatment (Milbemax®, as described below) and a general clinical examination was performed. The experiment accorded to the Swiss legislation on animal welfare and was authorised by the animal experiment commission.

2.2. Experimental design

The course of treatments and egg inoculations in the three experimental groups can be followed in Fig. 1. Each six-dog group consisted of both genders and three vertically *T. canis*-infected (subgroup a) and three helminth-free (subgroup b) dogs.

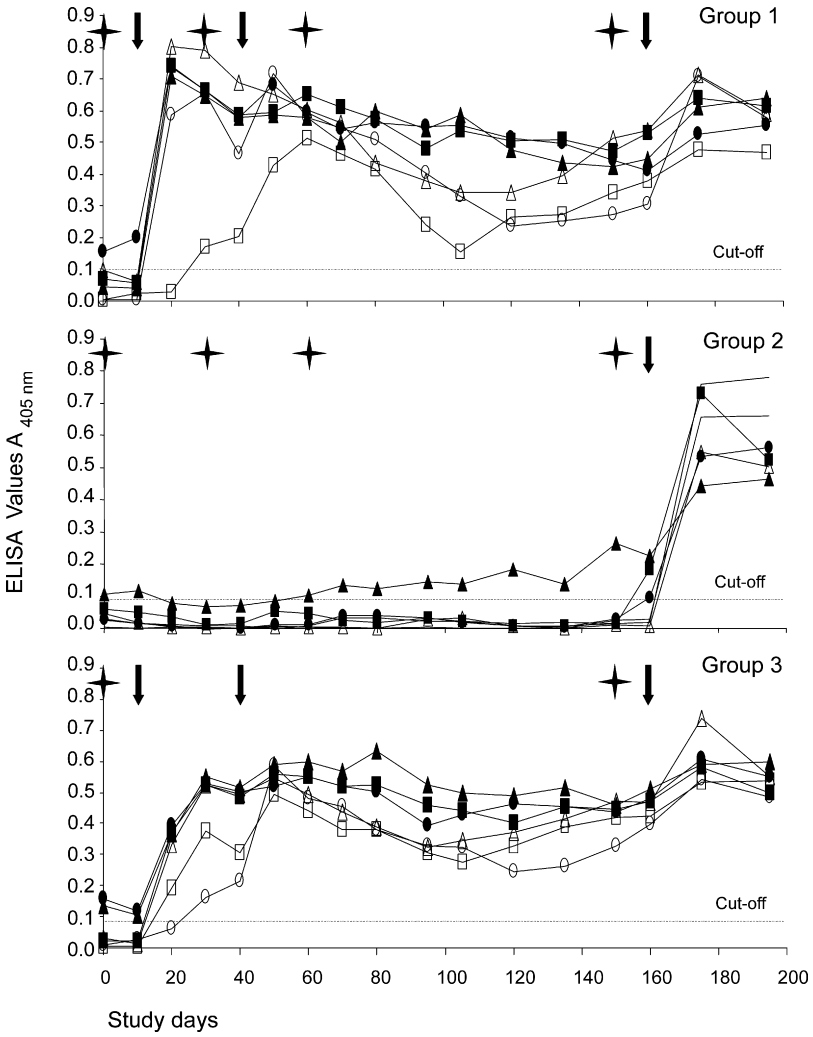

Fig. 1. Specific serum antibody detection (IgG) in ELISA against *T. canis* larval E/S antigen in three groups of six dogs in the course of experimental infection with 100 embryonated *T. canis* eggs (solid arrows) and anthelmintic treatment (radiating bodies). For all dogs, individual curves of ELISA values are shown: (a) solid symbols: dogs with history of vertically transmitted patent *T. canis* infections; (b) open symbols: dogs kept helminth-free prior to the beginning of the experiment. The cut-off was determined using 18 serum samples taken from the nine helminth-free dogs 2 and 3 months before study start, respectively.

On study day 0, all 18 dogs were treated once with Milbemax®. Dogs of group 1 were each administered 100 embryonated *T. canis* eggs (infections 1 and 2) on study days 10 and 40, respectively, and Milbemax® treatments followed on study days 30 and 60, respectively (Fig. 1). Dogs in group 2 were negative controls and got only Milbemax® treatments as described for group 1. Group 3 served as positive control group and got eggs as group 1, but without any anthelmintic treatment. In the following observation period from study days 60 to 150, none of the participating animals received any eggs or anthelmintic treatment. From study day 150 onwards, all groups were given piperazine citrate on 3 consecutive days, allowing the detection of excreted paralysed worms to prove or rule out intestinal infections. On study day 160, all 18 dogs were administered 100 embryonated eggs (infection 3) to investigate the establishment of new patent infections despite previous intensive exposure to the parasite (group 3), or after discontinuation of a regular frequent macrocyclic lactone treatment with or without parasite contact (groups 1 and 2, respectively). The infection was followed by observation until study day 220.

2.3. Blood and serum samples (Haematology, Serology)

Blood samples were drawn aseptically from the jugular vein with sterile needles and evacuated EDTA

tubes (Vacuette®, Greiner bio-one). Sampling took place in 10 days intervals during the first 80 study days and biweekly for the rest of the study.

At the Clinical laboratory at Vetsuisse Faculty, University of Zurich, differential blood cell counts were carried out in 15–30 days intervals. For specific antibody detection, a portion of the blood was centrifuged at 500 × *g* for 10 min and the plasma aliquotted and stored at −20 °C until it was used for ELISA.

2.4. Collection of faecal samples and coproscopic examination

Once a week, dogs were housed separately overnight and individual faecal samples were collected. A combined sedimentation-floatation technique as described (Eckert et al., 2005) with $ZnCl_2$-solution (density: 1.45) was carried out to identify patent infections by detection of *T. canis* eggs. In order to determine more exactly the prepatent periods, samples were examined 3 times/week from study day 190 (30 days post-infection 3) onwards.

2.5. Anthelmintic treatment

Milbemax® (Milbemycin oxime + Praziquantel, Novartis) tablets size M were used for anthelmintic treatment according to the manufacturer's specifications. According to body weights of the dogs, the administration of one tablet corresponded to 0.92–1.56 mg of milbemycin oxime/kg body weight. For treatment on day 150, Piperazine citrate (Piperazinzitrat Stricker, Werner Stricker AG, Zollikofen, Switzerland) was given with a dosage of 0.1 mg/kg on 3 consecutive days.

2.6. Embryonated T. canis eggs and infection

To ensure a successful infection, each administration of embryonated eggs took place 10 days after anthelmintic treatments to exclude high residual levels of active agents in the animals. *T. canis* eggs were extracted from the uteri of adult female *T. canis* worms from naturally infected beagle dogs kept in the institute's facilities. To embryonate, the eggs were treated as described (Annen et al., 1975) and stored in 0.1 N H_2SO_4 at 4 °C before use. For administration, eggs were washed in sterile phosphate buffered saline solution (PBS) until pH had reached 6. For every infection dose, 100 embryonated eggs were taken out with a glass pipette under the microscope and mixed with commercial moist cat food fed to the dogs.

2.7. Antibody detection ELISA

ELISA procedures were optimised with regard to E/S antigen (larval *T. canis* excretory/secretory antigen (de Savigny, 1975)) and conjugate dilutions. The ELISA was carried out as described by Deplazes et al. (1995), with the following modifications: E/S antigen was diluted in 0.1 M carbonate/bicarbonate coating buffer at 1:1000, and specific affinity purified, alkaline phosphatase conjugated goat anti-dog IgG(γ) antibodies (KPL, Gaithersburg, MD, USA) were used at a dilution of 1:800. All incubation steps were performed at 37 °C for 1 h. Substrate (4-nitrophenyl phosphate disodium salt hexahydrate (Fluka) in 0.05 M carbonate/bicarbonate plus 1 mM MgCl buffer) was applied and absorbance values at 405 nm (A_{405}, reference filter A_{630}) were read 23 min later in a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland). Before starting the test runs, several serum and plasma samples taken in a preliminary test (simultaneously from the same animals each) had been compared to ensure there was no significant difference between serum and plasma in ELISA results (data not shown). On every ELISA plate, we included negative control sera from laboratory dogs known to be free from nematodes and positive control sera of dogs with proven experimental *T. canis* infections (data not shown), as well as conjugate and background controls. For cut-off determination, we used samples taken from the nine *T. canis*-free dogs 2 and 3 months prior to study start. The cut-off was calculated as mean + threefold standard deviation of the absorbance values measured at 405 nm (A_{405}).

3. Results

3.1. Development of patent infections

Following infections 1 and 2, no eggs were found in the faeces of the dogs of groups 1 (infected and treated) and 2 (treated only) and consequently, no worms were recovered from their faeces following the piperazine treatment on study day 150. In the infected but not subsequently treated group 3, dogs with history of vertically transmitted patent infections (subgroup 3a) as well as previously *Toxocara*-free dogs (subgroup 3b) became patent. No differences concerning the prepatent periods of 42–56 days were found in these two subgroups (Table 1). Weekly faecal examinations revealed the ongoing patency. On study day 100 one dog of subgroup 3a and on study day 108 a dog of subgroup 3b spontaneously expulsed a part of the worm burden; however, continuous egg excretion persisted in

Table 1
Development of patent *T. canis* infections in three groups of dogs with each two subgroups: (a) prior *T. canis*-exposed or (b) prior helminth-free dogs

	Patent infections/number of dogs (prepatency in days)			
Groups/Subgroups*[1]	Infection *1*, study day 10	Infection *2*, study day 40	Infection *3*, study day 160	
Group 1a	0/3 (-)	0/3 (-)	0/3 (-)	} *[4]
Group 1b	0/3 (-)	0/3 (-)	1/3 (42)	
Group 2a	0/3 (-)*[2]	0/3 (-)*[2]	2/3 (40, 42)	} *[4]
Group 2b	0/3 (-)*[2]	0/3 (-)*[2]	3/3 (40, 45, 55)	
Group 3a	3/3 (42, 48, 56)	3/3*[3]	2/3 (45, 45)	
Group 3b	3/3 (48, 48, 51)	3/3*[3]	2/3 (43, 52)	

Dogs were infected 1–3 times with 100 embryonated *T. canis* eggs. Groups 1 and 2 were treated on study days 30 and 60, and all groups were treated on study day 150.
*[1](a) Dogs with history of vertically transmitted infections that developed to patency as pups; (b) helminth-free dogs that had no contact to *T. canis* until the beginning of the experiment. *[2]According to experimental design, no infection took place at the time point. *[3]Patent infection persisted from first infection until anthelmintic treatment on day 150. *[4]Significant difference ($p < 0.05$) between whole groups ($n = 6$).

all six animals during the whole observation period before treatment with piperazine on study day 150. After the piperazine doses, adult worms were recovered from the faeces of all dogs of group 3, and their faeces remained egg free subsequent to this deworming until infection 3 became patent.

Coproscopical examinations following infection 3 on study day 160 revealed the development of patent infections in dogs of all three groups, as displayed in Table 1. Patent infections were found in one dog of group 1, exposed 3 times to eggs but treated with Milbemax® after the two first infections. This animal belonged to the previously helminth-free subgroup 1b. In group 2, which had not been experimentally exposed to *T. canis* eggs until study day 160, two and three dogs of subgroups 'a' and 'b', respectively, became patent (Table 1). Finally, in group 3 which had presented patent infection between study days 52 and 150, two dogs of each subgroup showed patent infections again. Using Fisher's exact test to compare the results of infection 3, group 1, with one out of six animals becoming patent, was significantly different to group 2, with five out of six patent animals ($p = 0.04$ for a one tailed test). There were neither significant differences between group 1 and group 3 nor between group 2 and group 3. Prepatent periods of 40–55 days did not differ significantly between the groups or after first or repeated infections (for details, see Table 1). All animals with positive coproscopical results were taken out of the study, and five out of the eight animals that did not develop patent infections were followed up until 100 days post-third egg infection. During this time, no further patent infections developed.

3.2. Specific antibody detection

To document larval invasion or parasite free status during the study, all dogs were followed up in 10–14 days intervals for specific antibodies against larval E/S antigens. Fig. 1 shows specific antibody reactions (IgG) in the three experimental groups and subgroups. All dogs of the helminth-free subgroups initially showed ELISA values below the cut-off. However, four of the nine dogs with history of vertically transmitted patent *T. canis* infections showed low specific reactions at study day 0. Higher specific antibody responses could be detected 10 days post-infection 1 in all dogs of groups 1 and 3, whereas in group 1 values rose more sharply and reached a higher level by study day 20 than those of group 3. After study day 20, values of groups 1 and 3 converged to comparable levels. Antibody reaction levels of the uninfected dogs (group 2) remained significantly lower until day 160 of the study (Fig. 1).

After the infection on study day 160, moderately increased reactions were seen in the previously infected dogs (groups 1 and 3), and a sharp increase of specific reactions in the previously non-infected control group 2. One dog in group 2 suffered several times from fever

and leukocytosis during the study. Absorbance values of this animal always stayed visibly higher (Fig. 1, subgroup 2a) as the group mates' until study day 160 and increased less after the administration of embryonated eggs.

3.3. Blood cell counts

Before being experimentally infected, the dogs had not shown elevated eosinophilic counts at any time point (data not shown). After each egg administration, eosinophilic counts rose in all concerned dogs. Most met or exceeded the reference value (1.29×10^3 μl^{-1}) at 10 days post-infection (dpi), reaching highest counts about 20 dpi (up to 6.56×10^3 μl^{-1} in one group 2 dog on study day 175) and dropping to normal by 30 dpi. Eosinophilic counts did not differ in the subgroups 'a' and 'b' or in animals before or after reaching patency compared to animals that did not develop patent infections. Apart from increased eosinophils, there were no significant changes detectable in the haemogram of any dog throughout the study.

4. Discussion

The successful induction of patent *T. canis* infections in adult dogs with and without previous exposure to the parasite confirms the few earlier experimental data and several observations in prevalence studies. Our results support the hypothesis that the development of patent infections beyond puppy age is more than coincidence due to particular circumstances such as consumption of infected paratenic hosts, immunosuppression, or hormonal changes. The age of our dogs of 8–10 months at the beginning of the study was higher than the ''age resistance limit'' indicated by various authors (Webster, 1956; Greve, 1971). When challenged by infection 3 of the experiment, all dogs were older than a year. At this age, the canine immune system is regarded as fully competent (Felsburg, 2002).

To approximate the natural exposure of the canine population to *T. canis* eggs in the field, we chose ''low dose'' infections of just 100 embryonated eggs per animal as described earlier (Overgaauw, 1997). Actually, most experimentally induced intestinal infections in dogs employed a low number of eggs. Dubey (1978) induced patent intestinal infections in 24 of 25 three-month-old dogs fed 10–1000 embryonated eggs, but in none of 20 dogs administered 10,000 eggs. He also found patent infections in three out of six adult beagle dogs fed 100 eggs, but unlike our experiment, all of them were *T. canis*-naïve. Fernando (1968), Dubey (1978) and Glickman et al. (1981) delivered evidence that patent infections are less likely to develop after infections with higher doses of eggs. However, Webster (1956) published the establishment of infections in up to 6-month-old dogs after infection with large doses of eggs. Lloyd et al. (1981) had a comparable success, but only when treating a 6-month-old dog with corticosteroids. Additionally, the experimental animals used by the two latter authors were still younger than ours. In a reported case of repeated patent infections in three adult male greyhounds with unknown history of previous exposure and acquired immunity, the success of the experiment was restricted to the particular susceptibility of the breed and sex of the dogs and to the dose of infective inoculum (100–200 eggs/infection) (Maizels and Meghji, 1984). Partly, our results contradict this opinion as we induced patent infections in another dog breed and in both sexes. On the other hand, our results confirm these authors' opinion that the overall susceptibility of adult dogs to patent *T. canis* infections should be estimated at a higher level than so far accepted.

In our age matched experimental groups, previous exposure to vertical infections as well as experimentally induced patent infections had no effect on reestablishment of patent infections compared with helminth-free animals. However, the observed trend suggests that dogs with parasite contact and regular anthelmintic treatment (group 1) are less likely to develop patent infections following ingestion of eggs. This tendency of developing a higher resistance might be explained by a more pronounced immunological response against antigens released by the destroyed migrating larvae after milbemycin oxime treatment.

Increased specific antibody (IgG) reactions against larval E/S antigens and eosinophilic counts from 10 dpi on attest the infected dogs' immune reaction patterns after each inoculation with *T. canis* in our study. Anthelmintic treatment did not inhibit the development of characteristical immune mediated features in the treated groups (groups 1 and 2) following infection. This result implicates that the investigated antibody reactions and increased eosinophilic counts, even though proving immune functionality, are not necessarily associated with protection against patent infections.

The control dog (group 2a) that had shown higher specific reactions than its group mates and suffered from intermittent fever and leukocytosis during the study originated from the vertically infected subgroup. We never found any clues relating to the causality of these episodes. Necropsy of this dog (following euthanasia after study termination) did not reveal any

somatic damage, for instance by migrating larvae, neither.

Prepatent periods for *T. canis* differ depending on the route of infection, age of the host and inoculum size. Puppies infected *in utero* can start to shed eggs less than 3 weeks after birth (Lloyd et al., 1983). For infection via the lactogenic route, prepatency is about 4 weeks (Stoye, 1976). Male and female dogs infected by ingestion of paratenic hosts (mouse tissue containing *T. canis* larvae) developed patent worm infections 34–48 dpi (Herschel, 1981). Concerning oral infections with embryonated eggs, Dubey (1978) reported prepatencies of 32–35 days in experimentally infected puppies whereby he agrees with Glickman et al. (1981), who described the onset of patency as 31–34 dpi in 2-month-old beagles. Referring to the results of our study, and to other authors, prepatency seems to be slightly extended in older hosts. In 3-month-old dogs, Greve (1971) found adult worms just in the intestines of dogs which he kept for 35 or more days after infection. Saeed et al. (2005) produced experimental intestinal *T. canis* infections in 14-month-old silver foxes when administering them repeated doses of 400 embryonated eggs or larvae, respectively, and found prepatencies of 33–41 days. All in all, our results comprise 16 cases of onset of patency with *T. canis*, and all of them developed between 40 and 56 dpi. Prepatency did not seem to be influenced by previous *T. canis* exposure and immune response, respectively.

Another resistance-related feature observed in this study is expulsion of a part of the worm burden in two dogs, one at 60 and one at 68 days after the second low dose infection. This phenomenon has been described earlier as a response to superinfection (Fernando, 1968). Nevertheless, not all intestinal worms were eliminated from both concerned dogs, as their coproscopic examinations remained positive. Ongoing patency was proven during 90 days in the six dogs of group 3 until anthelmintic treatment. A patent period of this length accords to Schantz and Glickman (1981) who determined the average life span of *T. canis* worms to be 4 months.

Puppies are quantitatively regarded as the main shedders of *T. canis* eggs (Overgaauw, 1997; Lloyd, 1998). However, this group of dogs is more frequently treated with anthelmintics and their environment is more restricted. On the other hand, the epidemiological and zoonotic impact of adult dogs should be reconsidered. Increasing fox populations even in urban areas of central Europe (Deplazes et al., 2004; Reperant et al., 2007) with high *T. canis* prevalences up to more than 70% maintain a constant infection pressure for the dog population. Even if intestinal infections are not associated with clinical disease in adult dogs, based on the zoonotic potential, the guidelines of independent international expert committees such as CAPC for the United States (CAPC, 2007) or ESCCAP for Europe (ESCCAP, 2007) recommend that the risk of patent *Toxocara* infections in companion animals should be minimised by more frequent treatment or regular coproscopic testing. Protection against patent *T. canis* infections can be achieved theoretically by continuous anthelmintic treatment within the prepatent period. Consequentially, based on the prepatent period observed in this study, a regular treatment every 6 weeks would prevent most patent infections. A more rigid concept with monthly administration of macrocyclic lactones as *Dirofilaria immitis* prophylaxis suppresses nearly all canine nematodes, including patent *T. canis* infections (Deplazes, 2006). As shown in this study, such a continuous anthelmintic treatment seems not to decrease the resistance to further patent infections in dogs continuously exposed to *T. canis* eggs.

Acknowledgements

We kindly thank Prof. Hans Lutz and his laboratory staff (Clinical laboratory, Vetsuisse Faculty, University of Zurich, Switzerland) for the friendly communication and competent analysis of our blood samples. Furthermore, we thank the keepers, Armin Rüdemann and Esther Merz, for their great participation and support throughout the study.

This work represents the dissertation of Anna Sophie Fahrion, veterinarian.

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